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(54) Title: METHOD FOR THE PREPARATION OF A PROBE FOR NUCLEIC ACID HYBRIDIZATION

(57) Abstract

The invention relates to a method for preparing a probe, thus prepared probe, and the use of such a probe for selectively choosing sequence for nucleic acid diagnostic purposes, using preferably homogenous solutions. The invention is based upon a great number of probes having different sequences and lengths which all are complementary to different parts of the nucleic acid to be detected, which probes are synthetised on a solid matrix. The signal which they provide in non-hybridized condition is monitored, whereupon the nucleic acid to be detected is added, and the signal is monitored again. Those probes that show the most significant difference in signal are those, from a sensitivity point of view, that are the most suitable one.

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METHOD FOR THE PREPARATION OF A PROBE FOR NUCLEIC ACID HYBRIDIZATION.

DESCRIPTION

Technical field

The present invention relates to a method for the preparation of probes, in particular preparation of selective probes for the identification of nucleic acids; a matrix comprising such probes, and the use of such a probe for the hybridization of nucleic acids.

Background of the invention

Probes for the hybridization of nucleic acids are used to show the presence of a certain nucleic acid in a test solution. Traditional probes provide no appreciable differences concerning detectable features in free and hybridized conditions but the nucleic acid is determined separation of hybridized and non-hybridized probes (Gillespie & Spiegelman, J. Mol. Biol. 12, 829, 1956). Homogenous test methods, however, use probes the signals of which change at hybridization (Figure 1). Such probes are composed by a sequence-recognizing part (SID) and a reporting group (RG) (Figure 2) where SID as a rule is a synthetic oligodeoxy ribonucleic acid (Barton, J., US-A-5,157,032; Yamana et al., Nucl. & Nucl., 11 (2-4), 383, 1992; Linn et al., EP-A-0 710 668, US-A-5,597,696) or a nucleic acid analogue (Kubista, PCT/SE97/00953). RG is commonly a colouring agent the fluorescence of which increases at the binding to a nucleic acid. As shown by Nygren et al, Biopol., 46, 39-51 (1998) the properties of such colouring agents are heavily dependent on the sequence of the nucleic acid. This, as shown in PCT/SE97/00953, leads to the fact that both the background fluorescence of free probes as well as the fluorescence of the hybridized probes depend on the sequence of the SID and consequently of the target sequence (MS) selected, being complementary to SID. Further, it is probable that even the sequence closest to MS is of importance.

Nucleic acids can, as a rule, be determined selectively using a great number of probes which differ in the SID part by recognizing different MS which constitute unique segments of the nucleic acid. A nucleic acid having the length **m**, comprises **m+n+1** stretches of the length **n** which all are potential MS. As the length of MS has not to be particularly long in order to be

unique (a 15 bases long stretch appears as an average once per $4^{15} = 10^9$ bases) a great number of probes can be directed to a certain nucleic acid. A genome having 1000 bases, which is rather characteristic of a virus can be determined using 981 probes having the length of 20 bases, 982 of the length 19, 983 of the length 18, etc. Bacterial genomes which are considerably larger, can be determined using a still greater number of different probes. In order to determine a specific mutation the choice is more restricted as the sequence of the probe has to overlap the presumptive mutation, but still there are several alternatives.

In PCT/SE97/00953 one selects the sequence of SID starting from the knowledge of the known properties of RG, and for asymmetric cyanine dye stuffs SID's are proposed the terminal bases, preferably, mixed pyrimidines (-TT or -CT). This strategy comprises several restrictions. On one hand detailed studies of the properties of the dye stuff are required which can not be trivially extrapolated to the properties of the probes, due to differences in experimental conditions, etc, and on the other hand the sequence requirements as a rule of an indefinite number of sequences (1/8 of all sequences, e.g., end with either -TT or -CT). Finally, there is no consideration concerning the sequence closest to MS.

The present invention solves these problems. Basically, it is a method for determining which of a great number of potential probes to a certain nucleic acid, that has the lowest background signal and which of these that obtains the strongest signal at hybridization. The difference in determinations is the increase in signal strength which is obtained using the different probes.

Description of figures

Figure 1. Principle for homogenous testing

Figure 2. Probe for homogenous testing comprising a sequence recognizing part (SID) and a reporting group (RG)

Figure 3. Principle showing how the most suitable probe for a nucleic acid from a sensitivity aspect can be identified

A: Examples of probes which all recognize the same nucleic acid

B: These probes being synthetised onto a solid matrix

C: The fluorescence of probes monitored in free as well as hybridized conditions,

those providing the greatest increase in signal strength being the most suitable ones.

Figure 4 shows different probes and their different fluorescence intensities prior to hybridization

Figure 5 illustrates a construction of a probe described in an embodying example together with a sequence modification of the different probes the intensities of which are shown in Figure 4.

Description of the invention

Deoxyribonucleic acids and several nucleic acid analogues, such as peptide nucleic acids (PNA) are generally synthetised using a solid phase synthesis which i.a., allows the synthesis of a great number of fragments having different sequences and different lengths, as well, on a matrix (Khrapko et al., FEBS Lett. 256, 118. 1989; Southern, E., et al., Genomics 13, 1008, 1992; Caviani-Pease, et al., Proc. Natl. Acad. Sci., 91, 5022, 1994; Weiler et al., Nucl. Acids Res. 25, 2792, 1997). The fragments on this matrix can be hybridized using a labelled nucleic acid, e.g., fluorescent or radioactive groups, and the degree of hybridization can be determined from the signal of the nucleic acid. This technology has several applications and is often called DNA-chip technology.

In the present invention the DNA-chip technology is utilized in order to determine which of a great number of probes, which all, having a specificity enough, recognizes a certain nucleic acid suitable for nucleic acid hybridization, and then preferably in a homogenous solution. Probes, i.e., oligodeoxyribonucleic acids or nucleic acid analogues provided with reporting groups (RG) which all being complementary enough and specific enough to a certain nucleic acid are synthetised on a matrix (Figure 3). The signal, preferably fluorescence, which they give raise to, i.e., the background signal is monitored. Then a certain nucleic acid is added and the fluorescence, this time from the hybridized probes, are registered. The difference in signal strength at these determinations is the increase of signal strength obtained by the different probes. Those which show the greatest difference in signal strength are those from a sensitivity point of view being the most suitable ones.

Different matrix materials can be used, such as cellulose substrates, metal substrates and polymer substrates. When using metal substrates the metal is most often provided with a

coating of an amino acid to facilitate adherence. When using cellulose substrates a first nucleotide is attached to the substrate whereupon further nucleotides are synthetised onto the first one being attached until one has obtained a suitable nucleotide sequence.

The present probes can be used for the analysis of nucleic acid/s in the form of mRNA, DNA, PNA, PNA-PNA complexes, or DNA-PNA complexes.

The difference in signal strength at the hybridization can be obtained either by the probes changing its properties, signal difference non-hybridized condition visavi hybridized 'condition, or one hybridizes to a labelled nucleic acid using another reporting group, RG', whereby the difference in signal strength is obtained when the RG-group of the probe and the RG'-group of the target DNA approach each other. RG and RG' can the same or different. In a system comprising pyrene the fluorescence properties markedly when two pyrenes are brought into contact with each other, whereby an eximer fluorescence is obtained. Example of two different RG, RG' are energy transfer pairs such as fluorescein/tetramethyl rhodamine or fluorophore/quencher pair.

The invention will be described below with reference to an example illustrating the invention, without, however, being restricted thereto.

Example

Fifteen 10-bases long PNA-thiazole orange probes, complementary to different segments of the sequence GTCAGATGAGGAAGAGGCTATTGT, and a probe being complementary in a parallel orientation to the central polypurine region, were synthetised onto a Perspective-PP-NH_2-membrane (separated from the membrane with PEG-500-Glu-Lys-capronic acid linker) using an ABIMED ASP 222 Automated SPOT Robot (Weiler, J. et al., Nucleic Acids Res., **25**, 2792, (1997), Figure 1). The PNA monomers were attached as described by Weiler et al, supra. In the last step the thiazole orange dye stuff substituted with a carboxylic acid alkyl linker was activated and reacted in the same way as the Fmoc-PNA-monomers. After synthesis the side chain protecting groups were eliminated from the PNA oligomers by treatment using 90% TFA/5% of water/5% triethyl silane for 1 hr (TFA = trifluoro acetic acid).

The membrane was then moistened in a 10 mM borate buffer at pH 8.5 comprising an addition of 100 mM NaCl for 2 hrs, and was lightened using a standard UV-lamp having λ_{max} = 312 nm, and was photographed using a CCD camera (Figure 4). The background fluorescence of the probes have been expressed in relation to probe 16 (CCTCTTCCTC-TO), which exhibits the weakest intensity prior to hybridization and which, thereby, is expected to provide the greatest difference in signal strength after hybridization.

CLAIMS

1. Method for preparing probes comprising a sequence recognizing part (SID) and a reporting group (RG), which probes are complementary enough to different parts of a certain nucleic acid,
characterized in
that oligodeoxyribonucleic acids or nucleic acid analogues are attached to a solid matrix.

2. Method according to claim 1,
characterized in
that oligodeoxyribonucleic acids or nucleic acid analogues are synthetised on the solid matrix.

3. Method according to claim 2,
characterized in
that oligodeoxyribonucleic acids or nucleic acid analogues are stepwise synthetised on the solid matrix.

4. Method according to claim 1,
characterized in
that oligodeoxyribonucleic acids or nucleic acid analogues are attached to a solid matrix by means of adsorption.

5. Method according to claim 1,
characterized in
that the signal provided by RG is a fluorescence signal.

6. Method according to one or more of claims 1-4,
characterized in
that SID is a deoxyribonucleic acid, a deoxyribonucleic acid derivative or a deoxyribonucleic acid analogue.

7. Method according to claim 6,

characterized in

that SID is a peptide nucleic acid (PNA) or a peptide nucleic acid (PNA)-derivative.

8. Method according to one or more of claims 1-7,
characterized in
that the probe, comprising SID and RG is designed to change its properties at hybridization to a target sequence.

9. Method according to one or more of claims 1-8,
characterized in
that the probe, comprising SID and RG, is designed to provide a difference in signal strength by means of its RG at the hybridization to a target sequence.

10. Method according to one or more of claims 1-8,
characterized in
that the probe comprising SID and RG is designed to hybridize to a labelled target sequence comprising a reporting group RG' being equal to or different from RG of the probe whereby a difference in signal strength is provided to develop when RG and RG' approach each other at the hybridization.

11. Method according to one or more of claims 1-10,
characterized in
that the matrix consists of a cellulose substrate, a metal substrate or a polymer substrate or a mixed substrate of these.

12. Matrix for a probe for nucleic acid hybridization, comprising oligodeoxyribonucleic acids or nucleic acid analogues which comprise a sequence recognizing part (SID) and a reporting group (RG),
characterized in
that said oligodeoxyribonucleic acids or nucleic acid analogues are fixedly attached to the matrix and are complementary enough to different pars of a certain nucleic acid.

13. Method according to claim 12,
characterized in
that the matrix consists of a cellulose substrate, a metal substrate or a polymer substrate or a mixed substrate of these.

14. The use of probes consisting of SID and RG where the detectable properties of the probe are changed at hybridization to a nucleic acid, and the sequence of the SID is selected by comparing the signal in free and hybridized condition of at least 10, preferably at least 100, and more preferably at least 1000 probes, which hybridize to different parts of a certain nucleic acid, for the detection of nucleic acid by means of hybridization, whereby the probes are synthetised to a solid matrix and whereby their signals are determined in the absence of a certain nucleic acid and/or their signals are determined in the presence of a certain nucleic acid.

15. Use according to claim 14,
wherein the hybridization takes place in a homogenous solution.

16. Use according to one or more of claims 14-15,
characterized in
that the probe, comprising SID and RG, is designed to change its properties at hybridization to a target sequence.

17. Use according to one or more of claims 14-16,
characterized in that the probe, comprising SID and RG is designed to provide a difference in signal strength by means of its RG at the hybridization to a target sequence.

18. Use according to one or more of claims 14-16,
characterized in
that the probe, comprising SID and RG is designed to hybridize to a labelled target sequence comprising a reporting group RG' being equal to or different from RG of the probe, whereby a difference in signal strength is provided to occur when RG and RG' approach each other at hybridization.

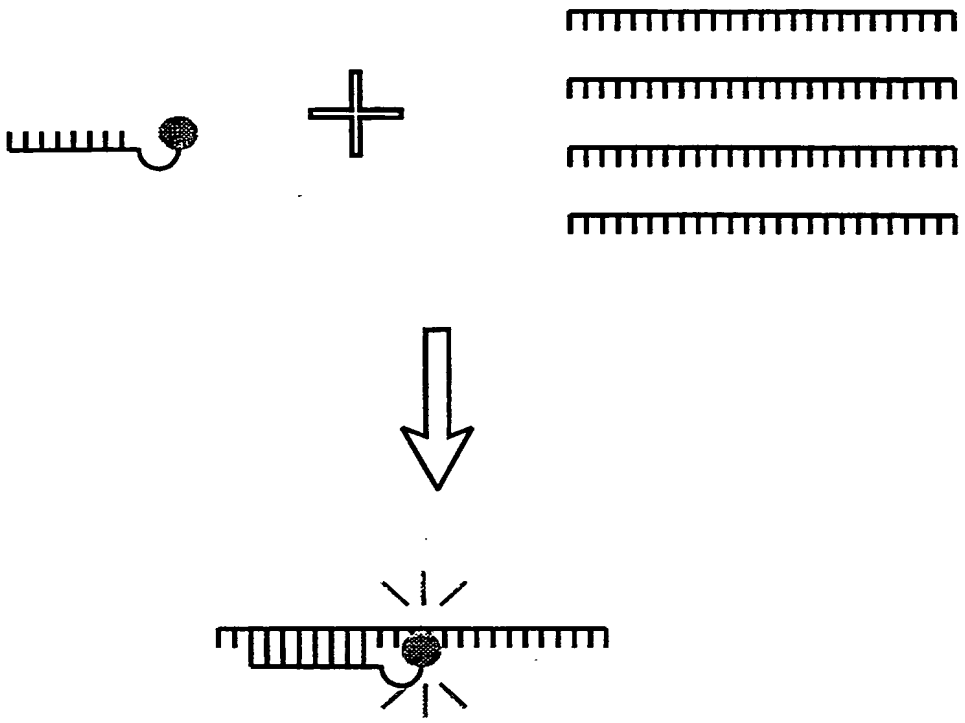


Figure 1

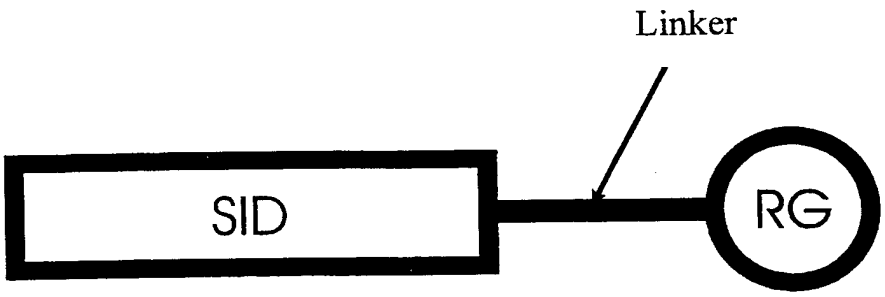


Figure 2

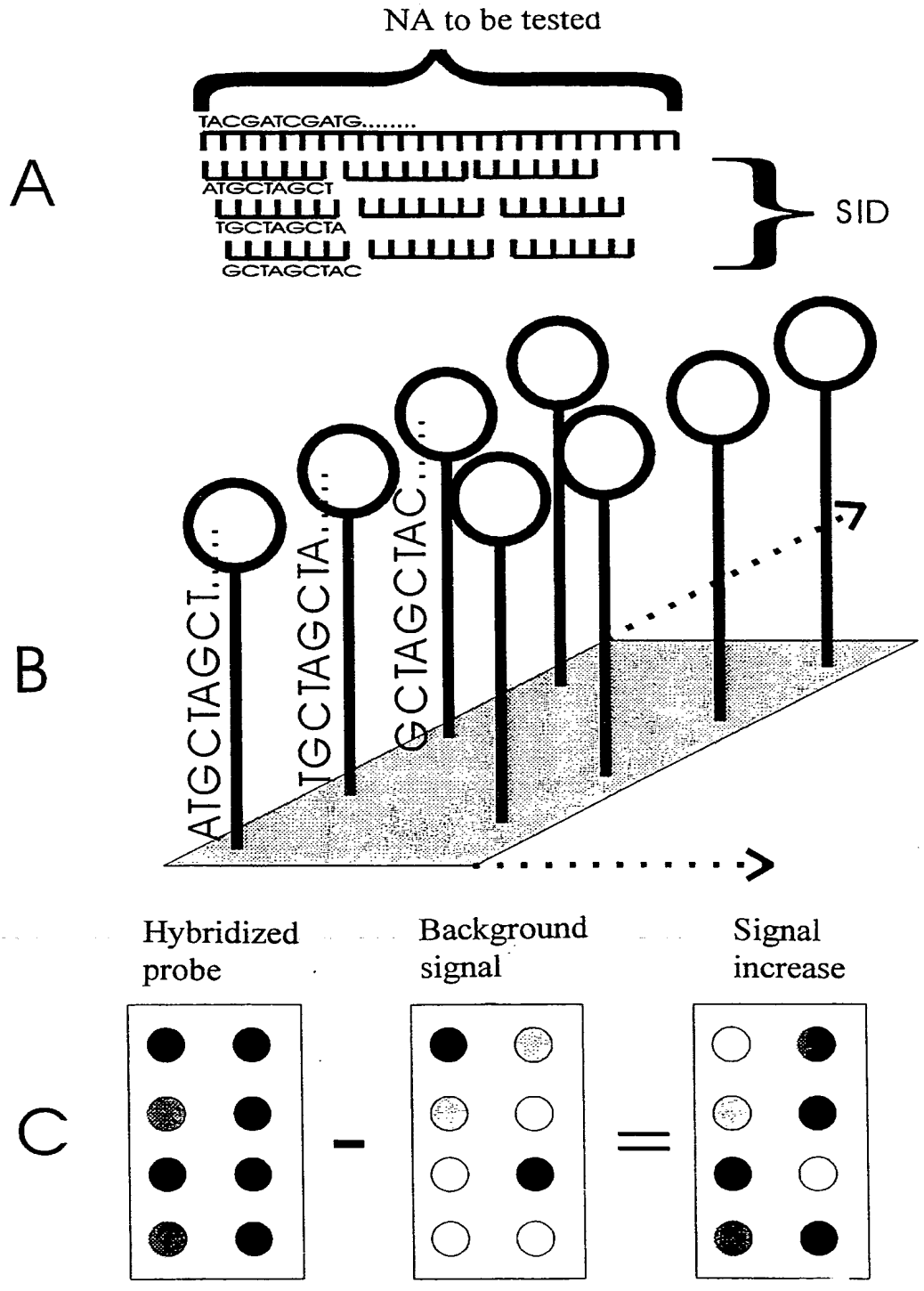

NA to be tested
A
TACGATCGATG.....
ATGCTAGCT
TGCTAGCTA
GCTAGCTAC
SID
B
ATGCTAGCT...
TGCTAGCTA...
GCTAGCTAC...
C
Hybridized probe
Background signal
Signal increase

Figure 3

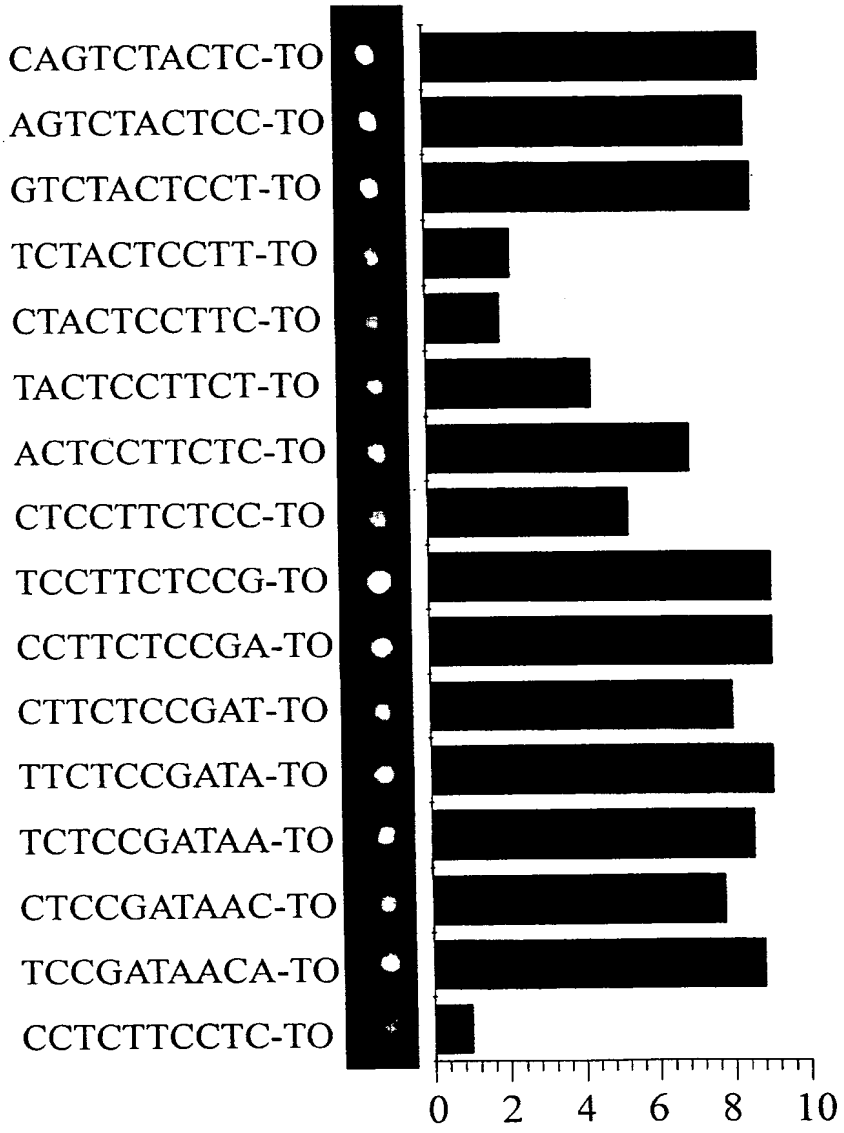
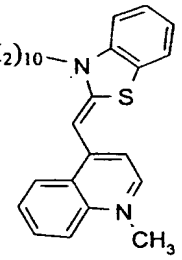

CAGTCTACTC-TO
AGTCTACTCC-TO
GTCTACTCCT-TO
TCTACTCCTT-TO
CTACTCCTTC-TO
TACTCCTTCT-TO
ACTCCTTCTC-TO
CTCCTTCTCC-TO
TCCTTCTCCG-TO
CCTTCTCCGA-TO
CTTCTCCGAT-TO
TTCTCCGATA-TO
TCTCCGATAA-TO
CTCCGATAAC-TO
TCCGATAACA-TO
CCTCTTCCTC-TO
0
2
4
6
8
10

Figure 4

Probe construction

Membrane-PEG-500-GLU-LYS-Capronic acid-PNA-CO-$(CH_2)_{10}$-



Thiazole orange (TO)

PNA Sequences

1	C	A	G	T	C	T	A	C	T	C											
2		A	G	T	C	T	A	C	T	C	C										
3			G	T	C	T	A	C	T	C	C	T									
4				T	C	T	A	C	T	C	C	T	T								
5					C	T	A	C	T	C	C	T	T	C							
6						T	A	C	T	C	C	T	T	C	T						
7							A	C	T	C	C	T	T	C	T	C					
8								C	T	C	C	T	T	C	T	C	C				
9									T	C	C	T	T	C	T	C	C	G			
10										C	C	T	T	C	T	C	C	G	A		
11											C	T	T	C	T	C	C	G	A	T	
12												T	T	C	T	C	C	G	A	T	A
13													T	C	T	C	C	G	A	T	A
14														C	T	C	C	G	A	T	A
15															T	C	C	G	A	T	A
16								C	C	T	C	T	T	C	C	T	C				

1																					
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16																					

Figure 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 98/01580

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12Q 1/68, C07H 21/00, C12N 15/10
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07H, C12Q, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9518136 A1 (ISIS PHARMACEUTICALS, INC.), 6 July 1995 (06.07.95), page 16, lines 5-6, page 18, line 30 --	1-13
X	WO 9705156 A1 (BEHRENS, CARSTEN), 13 February 1997 (13.02.97), page 3, line 17-21, claims 28, 30 --	1-13
X	WO 9511912 A2 (BECKMAN INSTRUMENTS, INC.), 4 May 1995 (04.05.95), page 19, line 28, claim 17 and the whole document --	1-13

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means
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"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 February 1999	14 -02- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 98/01580

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9211388 A1 (ABBOTT LABORATORIES), 9 July 1992 (09.07.92)	1-13
A		14-18
	--	
X	US 5646267 A (WOJCIECH J. STEC ET AL), 8 July 1997 (08.07.97), column 10, line 36-39 and the whole document	1-13
	--	
A	EP 0710668 A2 (BECTON DICKINSON AND COMPANY), 8 May 1996 (08.05.96)	1-13
X		14-18
	--	
X	EP 0714986 A1 (TOSOH CORPORATION), 5 June 1996 (05.06.96)	14-18
	--	
X	DE 3446635 A1 (CALIFORNIA INSTITUTE OF TECHNOLOGY), 27 June 1985 (27.06.85)	14-18
A		1-13
	--	
A	EP 0742287 A2 (MCGALL, GLENN H. ET AL), 13 November 1996 (13.11.96)	14-18
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 98/01580

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 98/01580

According to PCT Rule 13.2 an international application shall relate to one invention only or a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art.

The claimed invention relates to a method for producing a probe for a target nucleic acid having sequence recognising element (SID) covalently bound to a reporter group(RG) on solid support. Moreover the present application claims the use of a probe comprising SID and RG for detection of a nucleic acid by hybridisation. A special technical feature as required could have been either the probe per se, or the synthesis of the probe on solid support, however both these features are known in the art, see the search report e.g. WO95/18136 or EP0710668. Thus, the claimed invention fails to comply with PCT Rule 13.2 *a posteriori*.
The following inventions were found:
Invention 1, claims 1-13: a method for producing a probe for a target nucleic acid having sequence recognising element (SID) covalently bound to a reporter group(RG) on solid support.
Invention 2, claims 14-18: use of a probe comprising SID and RG for detection of a nucleic acid by hybridisation.

INTERNATIONAL SEARCH REPORT

Information on patent family members

21/12/98

International application No.
PCT/SE 98/01580

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9518136 A1	06/07/95	EP 0737201 A	16/10/96
		US 5541307 A	30/07/96
WO 9705156 A1	13/02/97	AU 6514096 A	26/02/97
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WO 9211388 A1	09/07/92	AT 163201 T	15/02/98
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